Research Article

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Rutaecarpine Modulates IL-1β and iNOS Expression in LTA-Induced Macrophages: A Confocal Microscopy Analysis

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ABSTRACT

Macrophages are essential immune cells involved in maintaining physiological homeostasis and mediating pathological responses. Activation of macrophages by lipoteichoic acid (LTA) from Gram-positive bacteria triggers inflammatory pathways, leading to the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β) and inducible nitric oxide synthase (iNOS). Understanding the regulation of these pathways is crucial for developing therapeutic strategies for inflammatory diseases. Our previous study has demonstrated that rutaecarpine (Rut), an alkaloid from the traditional Chinese medicinal herb Evodia rutaecarpa, can modulate inflammatory responses in macrophages. This study further investigates the effects of Rut on LTA-induced activation of RAW 264.7 macrophages, focusing on cell morphology, IL-1β secretion, and iNOS expression. Cell morphology was observed using a phase-contrast microscope, and IL-1β and iNOS expressions were assessed using immunofluorescence staining and confocal microscopy. The results showed that Rut treatment did not alter the morphology of RAW 264.7 cells at concentrations up to 20 μM. At 20 μM, Rut significantly inhibited LTA-induced IL-1β secretion and iNOS expression in macrophages. Confocal microscopy revealed that LTA stimulation markedly increased IL-1β and iNOS fluorescence intensities, which were significantly reduced by Rut pre-treatment. These findings suggest that Rut effectively inhibits LTA-induced inflammatory responses in RAW 264.7 macrophages by reducing IL-1β secretion and iNOS expression without affecting cell morphology, highlighting its potential as a therapeutic agent for treating inflammatory diseases associated with Gram-positive bacterial infections.

Keywords

Macrophages, Rutaecarpine, Inflammatory response, Lipoteichoic acid (LTA).

Introduction

Macrophages are pivotal immune cells that play crucial roles in both physiological homeostasis and pathological conditions. They are involved in processes such as tissue remodeling, wound healing, and the clearance of cellular debris. In pathological states, macrophages become activated in response to various stimuli, leading to a range of immune responses [1]. This activation can be beneficial, such as in host defense against infections, or detrimental, contributing to chronic inflammation and tissue damage. The phenotypic plasticity of macrophages allows them to adopt either pro-inflammatory (M1) or anti-inflammatory (M2) states, depending on the microenvironmental cues they encounter [2]. Understanding the mechanisms underlying macrophage activation is essential for developing therapeutic strategies for inflammatory diseases.

Lipoteichoic acid (LTA) is a major component of the cell wall of Gram-positive bacteria and acts as a potent activator of macrophages. When macrophages are exposed to LTA, it triggers a cascade of signaling pathways that result in the production

of various inflammatory mediators [3]. LTA binds to Toll-like receptor 2 (TLR2) on the surface of macrophages, initiating intracellular signaling that leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) [4]. These pathways collectively enhance the expression of pro-inflammatory cytokines and enzymes, contributing to the inflammatory response. Studying the impact of LTA on macrophages is crucial for understanding the host immune response to Gram-positive bacterial infections.

Interleukin-1 beta (IL-1β) is a key pro-inflammatory cytokine produced by activated macrophages [5]. It plays a significant role in mediating the inflammatory response and is involved in various pathological conditions such as autoimmune diseases, infections, and cancer. IL-1β is synthesized as an inactive precursor (pro-IL-1β) and requires cleavage by the inflammasome complex to become active [6]. Once activated, IL-1β exerts its effects by binding to the IL-1 receptor on target cells, promoting the expression of additional inflammatory mediators, enhancing the recruitment of immune cells to the site of inflammation, and inducing fever. The regulation of IL-1β production and activity is critical for maintaining immune homeostasis and preventing excessive inflammation.

Inducible nitric oxide synthase (iNOS) is an enzyme that catalyzes the production of nitric oxide (NO) from L-arginine in response to inflammatory stimuli [7]. In macrophages, iNOS is upregulated upon activation, leading to the generation of large amounts of NO, a reactive nitrogen species with antimicrobial and pro-inflammatory properties [8]. NO produced by iNOS plays a dual role in immune defense: it can directly kill or inhibit the growth of pathogens and modulate the function of various immune cells [9]. However, excessive production of NO can also contribute to tissue damage and exacerbate inflammatory diseases [10]. Therefore, the regulation of iNOS expression and activity is a critical aspect of the macrophage-mediated inflammatory response.

The interplay between LTA, IL-1β, and iNOS in macrophages is complex and crucial for the regulation of the inflammatory response [11]. LTA-induced activation of macrophages through TLR2 signaling results in the upregulation of both IL-1β and iNOS [12]. The production of IL-1β further amplifies the inflammatory response by inducing the expression of other cytokines and chemokines, while iNOS-mediated NO production can act as a signaling molecule influencing various cellular processes. The simultaneous upregulation of IL-1β and iNOS by LTA highlights the coordinated response of macrophages to Gram-positive bacterial components [13]. Understanding this relationship is vital for developing strategies to modulate macrophage activation and mitigate inflammatory diseases.

Rutaecarpine (Rut, Figure 1) is an alkaloid extracted from the fruit of Evodia rutaecarpa, a traditional Chinese medicinal herb. It has been shown to possess various pharmacological properties, including anti-inflammatory, anti-oxidative, and anti-cancer activities [14]. Recent studies have highlighted the potential of Rut in modulating inflammatory responses. It exerts its anti-inflammatory effects by inhibiting key signaling pathways involved in inflammation, such as NF-κB and MAPKs. In the context of macrophage activation, Rut has been demonstrated to reduce the production of pro-inflammatory cytokines and enzymes, thereby mitigating the inflammatory response [15]. Given these properties, Rut represents a promising therapeutic agent for treating inflammatory diseases induced by microbial components such as LTA.

Materials and Methods

Chemicals and Reagents

Rut (>98%) was purchased from Cayman Chem (Ann Arbor, MI, USA). Dimethyl sulfoxide (DMSO) and LTA were obtained from Sigma (St. Louis, MO, USA). Anti-iNOS polyclonal antibody (pAb) were purchased from Proteintech (IL, USA). Abcam (MA, USA) provided anti-IL-1β pAb and DAPI. CFTM488A Dye was obtained from Biotium (Hayward, CA, USA). Fetal bovine serum (FBS, 26140079), Dulbecco's modified Eagle medium (DMEM; 11965084), and L-glutamine penicillin/streptomycin (10378016) were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Culture

RAW 264.7 cells were purchased from ATCC (ATCC number: TIB-71). Cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin G and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were incubated at a density of 1×10^5 cells per well and then were treated with various concentrations of Rut (5–20 μM) or solvent control (0.1% DMSO) for 20 min. They were stimulated with LTA (10 μg/mL) or left unstimulated for 24 h [16]. Microscope images were taken at 20× magnification by an inverted contrast phase light microscope (Nikon, Tokyo, Japan).

Immunofluorescence Staining Assay

DMSO or Rut pretreatment following LTA-induced macrophages were carefully on coverslips, followed by fixation in a solution containing 4% (v/v) paraformaldehyde for 10 min. After fixation, macrophages underwent permeabilization using 0.1% Triton X-100 and were subsequently incubated in a 5% BSA solution in phosphate-buffered saline (PBS) for 30 min to block nonspecific binding sites effectively. Following this preparation, macrophages were subjected to immunostaining with specific primary antibodies targeting the proteins of interest over a 24-hour period. After immunostaining, thorough washing with PBS was carried out, and the macrophages were then exposed to secondary antibody (Alexa Fluor® 488-labeled goat anti-rabbit IgG) for an additional hour. Macrophages were then washed ×3 in PBS before mounting on glass slides using Flouroshield containing DAPI. Finally, imaging of the platelets was conducted using a confocal microscope (Leica TCS SP5, Mannheim, Germany) equipped with a 100× oil immersion objective [16].

Statistical Analysis

Data are expressed as the means ± standard error (SEM). Each

experiment was repeated at least four times. Statistical analysis was performed using a one-way analysis of variance (one-way ANOVA). Significant differences among the group were compared using the Newman–Keuls method. The p-values < 0.05 were considered statistically significant.

Results

Rut on Morphology in Raw264.7 cells

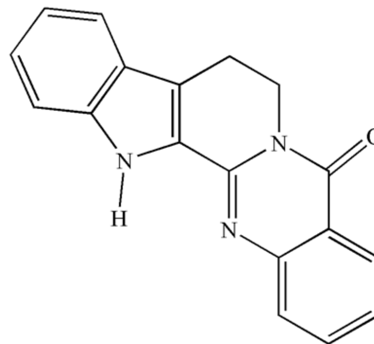
Cell morphology was examined to assess the inhibition of Rut on LTA-induced RAW 264.7 cells. Administration of RAW cells to 5, 10, and 20 μM Rut for 30 min and then stimulated with LTA (10 μg/mL) for 24 h did not alter cell morphology (Figure 2). Therefore, the concentrations of 20 μM Rut was used for the consequent analysis.

Rut Inhibits IL-1β Secretion in LTA-stimulated Macrophages

To further investigate the impact of Rut on IL-1β secretion, macrophages were pre-treated with 20 μM Rut before stimulation with LTA. Confocal scanning fluorescence microscopy revealed that LTA stimulation significantly increased the fluorescence intensity of IL-1β (green) compared to control macrophages (Figure 3A). This increase was markedly reduced in the presence of 20 μM Rut. The nuclear fluorescence intensity (blue) remained consistent across all experimental groups (Figure 3A-B). Statistical analysis of these fluorescence intensities is presented in the right panels of Figure 3B.

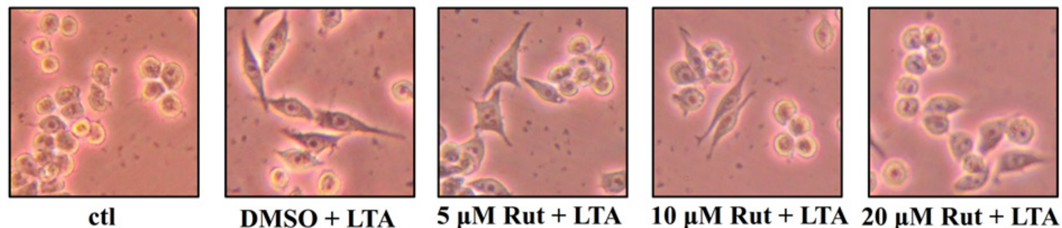
Rut Modulates iNOS Expression in LTA-Stimulated Macrophages

Given that IL-1β can induce iNOS expression and subsequently enhance nitric oxide production [17], we aimed to investigate if Rut could modulate this pathway in LTA-stimulated macrophages. Macrophages were pre-treated with 20 μM Rut followed by LTA stimulation to explore the effect of Rut on iNOS expression.



(Rutaecarpine, $C_{18}H_{13}CN_3O$, molecular weight: 287.32)

Figure 1: Chemical Structure of Rut.



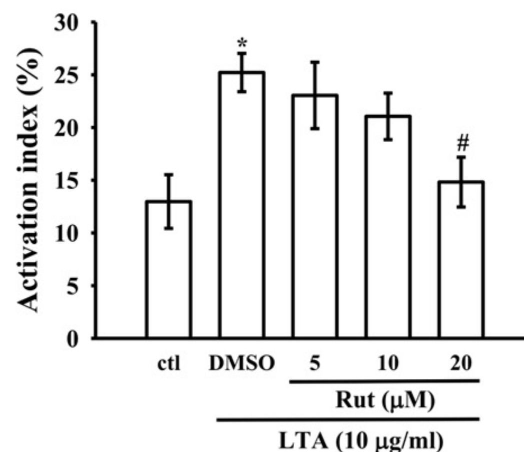
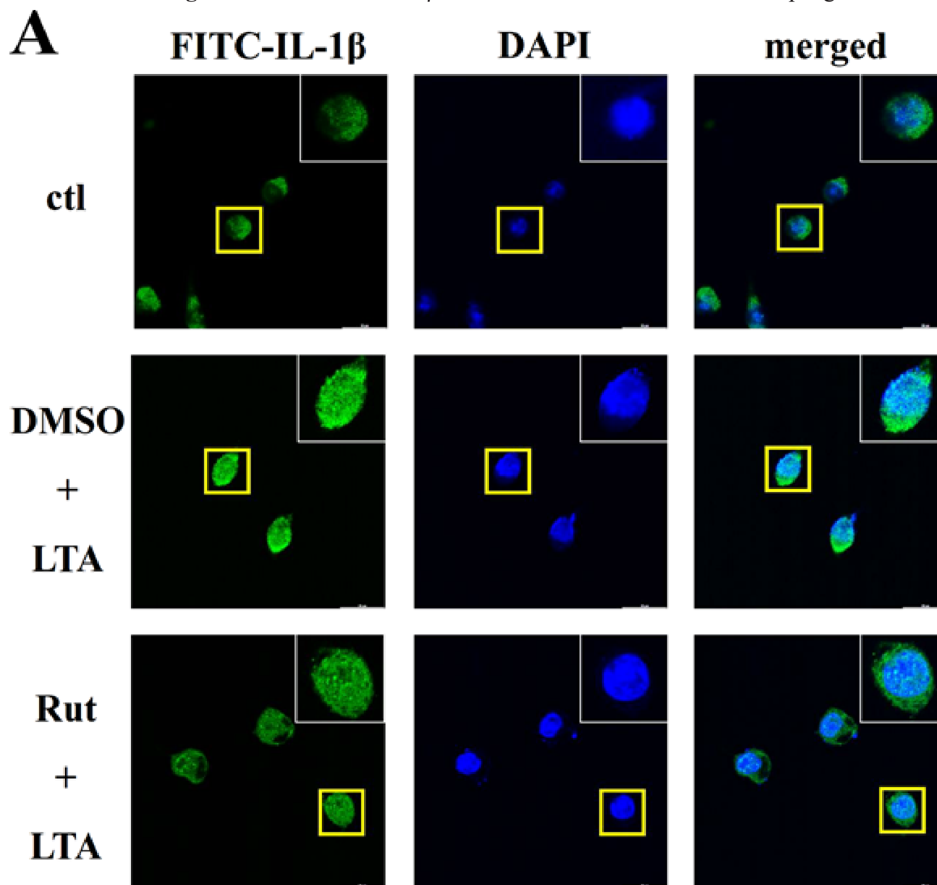
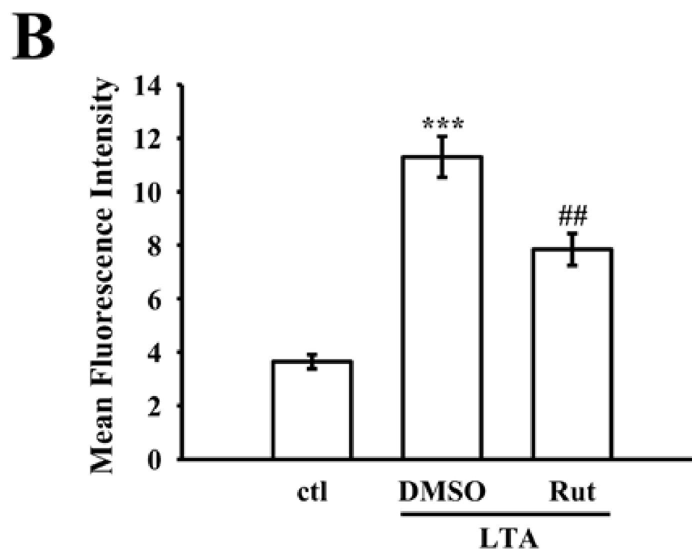


Figure 2: Effects of Rut on the Morphology of RAW 264.7 Cells.

RAW 264.7 cells were treated with 5, 10, and 20 μM Rut for 20 minutes followed by stimulation with LTA (10 μg/mL) for 24 hours. Cell morphology was observed under a microscope. Representative images are shown. Statistical analysis of the activation index (%) was performed, with significant differences indicated by p-values (*$p < 0.05$ compared to control, #$p < 0.05$ compared to LTA group).

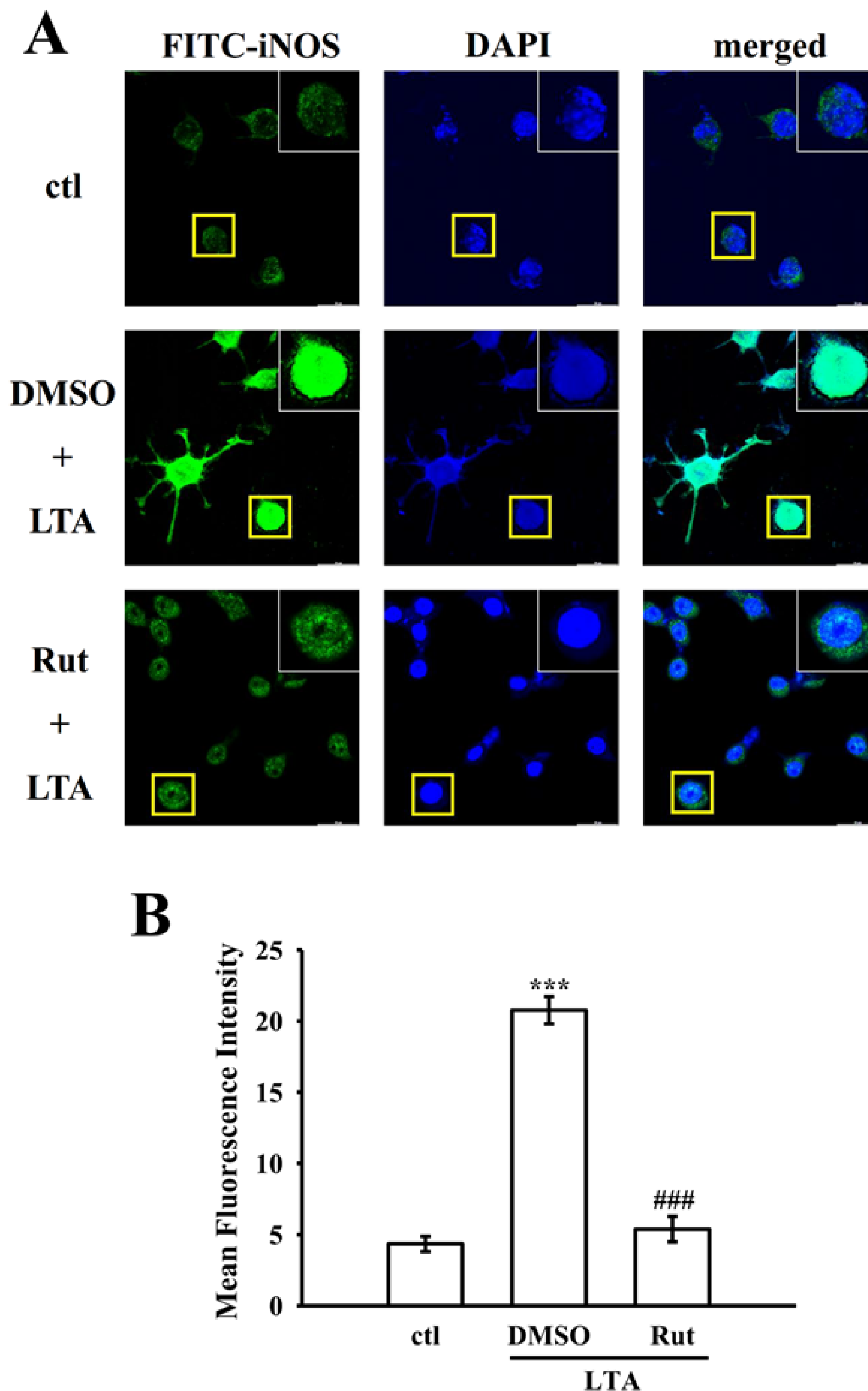
Figure 3: Rut Inhibits IL-1β Secretion in LTA-Stimulated Macrophages.





(A) RAW 264.7 macrophages were pre-treated with 20 μM Rut for 20 minutes before stimulation with LTA (10 μg/mL) for 24 hours. IL-1β expression was visualized using confocal scanning fluorescence microscopy. IL-1β is shown in green, and nuclear fluorescence is shown in blue. Representative images are shown. (B) Quantitative analysis of fluorescence intensities of IL-1β and nuclei. Data are presented as mean ± SEM. Significant differences are indicated by p-values (***$p < 0.001$ compared to control; ##$p < 0.01$ compared to LTA group).

Figure 4: Rut Modulates iNOS Expression in LTA-Stimulated Macrophages.



(A) RAW 264.7 macrophages were pre-treated with 20 μM Rut for 20 minutes followed by stimulation with LTA (10 μg/mL) for 24 hours. iNOS expression was visualized using confocal microscopy. iNOS is shown in green, and nuclei are stained with DAPI (blue). Representative images are shown. (B) Quantitative analysis of iNOS fluorescence intensity. Data are presented as mean ± SEM. Significant differences are indicated by p-values (***$p < 0.001$ compared to control; ###$p < 0.001$ compared to LTA group).

Confocal microscopy was employed to visualize iNOS (green) and nuclei (blue) in macrophages (Figure 4A). In control cells, iNOS was scarcely detectable. LTA stimulation significantly upregulated iNOS expression, as evidenced by the increased green fluorescence. Conversely, Rut pre-treatment markedly reduced the fluorescence intensity of iNOS in LTA-stimulated macrophages, suggesting an inhibitory effect of Rut on iNOS induction (Figure 4B).

Discussion

The current study elucidates the anti-inflammatory effects of Rut in LTA-induced RAW 264.7 macrophages, focusing on cell morphology, IL-1β secretion, and iNOS expression. Our findings provide significant insights into the potential mechanisms by which Rut modulates inflammatory responses in macrophages.

Our morphological analysis of RAW 264.7 cells has revealed that Rut does not induce significant morphological changes, even at concentrations as high as 20 μM. This observation strongly suggests that Rut exhibits non-toxic properties at these concentrations, rendering it a promising candidate for further investigation in pharmacological studies. The preservation of cell morphology is of paramount importance as it ensures that any observed biochemical alterations are not attributed to cytotoxic effects. Our findings are consistent with prior research indicating that Rut has minimal impact on cell viability [16]. This consistency in results underscores the safety profile of Rut and reinforces its potential as a therapeutic agent. The absence of significant morphological changes further supports its suitability for extended treatments and combination therapy strategies. The ability of Rut to maintain cell morphology and viability is in line with its potential applications across various therapeutic contexts. The absence of adverse morphological effects bolsters its candidacy for long-term treatments and combinatory therapeutic approaches.

The inhibition of IL-1β secretion by Rut in LTA-stimulated macrophages is a key finding of this study. IL-1β is a critical pro-inflammatory cytokine that plays a pivotal role in the amplification of inflammatory responses. LTA, a component of Gram-positive bacteria, is known to activate macrophages via TLR2, leading to the production of IL-1β. Our confocal microscopy results show that Rut significantly reduces the fluorescence intensity of IL-1β, indicating a decrease in its secretion. This suggests that Rut interferes with the TLR2 signaling pathway, thereby attenuating the production of IL-1β. This finding is in line with previous studies demonstrating the anti-inflammatory properties of Rut, further supporting its potential as a therapeutic agent for inflammatory diseases [15,16].

Additionally, Rut was found to modulate the expression of iNOS in LTA-stimulated macrophages [16]. iNOS is an enzyme responsible for the production of NO, a molecule with both antimicrobial and pro-inflammatory properties. Our study shows that LTA significantly upregulates iNOS expression, while Rut pre-treatment markedly reduces this upregulation. This indicates that Rut can inhibit the LTA-induced activation of the iNOS pathway, potentially reducing excessive NO production and preventing tissue damage associated with chronic inflammation. These results are consistent with existing literature that highlights the role of Rut in modulating NO production and iNOS expression [15,16].

Conclusion

The interplay between LTA, IL-1β, and iNOS underscores the complex regulatory mechanisms governing macrophage activation. By inhibiting both IL-1β secretion and iNOS expression, Rut demonstrates a broad-spectrum anti-inflammatory effect, likely mediated through the suppression of TLR2 signaling pathways. This dual inhibition addresses both cytokine production and enzyme activation, providing a comprehensive approach to mitigating inflammation. These findings suggest that Rut holds significant promise as a therapeutic agent for treating inflammatory diseases driven by Gram-positive bacterial infections.

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